Available online at www.sciencedirect.com
SCIENCE DIRECT
Peptides 26 (2005) 2151–2156
PEPTIDES
www.elsevier.com/locate/peptides

Mass spectrometric assignment of Leu/Ile in neuropeptides from single neurohemal organ preparations of insects

Ronald J. Nachman [a,*], William K. Russell [b], Geoffrey M. Coast [c], David H. Russell [b], Reinhard Predel [a,d]

[a] *Areawide Pest Management Research, Southern Plains Agricultural Research Center, USDA, 2881 F/B Road, College Station, TX 77845, USA*
[b] *The Laboratory for Biological Mass Spectrometry, Department of Chemistry, Texas A&M University, College Station, TX 77843, USA*
[c] *School of Biological and Chemical Sciences, Birkbeck College, London WC1E 7HX, UK*
[d] *Saxon Academy of Sciences, Research Group Jena, Erbertstrasse 1, Jena 07743, Germany*

Received 11 March 2005; received in revised form 27 April 2005; accepted 28 April 2005
Available online 21 July 2005

Abstract

Matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF–TOF) tandem mass spectrometry has been applied for the first time on an insect/arthropod target, focusing on PVK/CAP2b neuropeptides in the housefly *Musca domestica* and flesh fly *Neobellieria bullata*. The peptidomic analysis of single neurohemal organ preparations allows the unambiguous assignment of internal Leu/Ile positions not distinguishable by previous mass spectrometric techniques. The confirmation of side-chain fragments which allows assignment of Leu/Ile even from samples as small as neurohemal organs will greatly accelerate the identification of novel neuropeptides that are implicated in the regulation of critical physiological processes in insects. The unnatural Ile analog is 4.5 times more active than the native Leu sequence in a housefly Malpighian tubule fluid secretion assay, which reinforces the caveat that potency values in a biological assay cannot be relied upon to predict the native sequence.

Keywords: MALDI-TOF/TOF mass spectrometry; Insect; Neuropeptide; Periviscerokinin; CAP2b; *Musca domestica*; *Neobellieria bullata*; Peptidomics

1. Introduction

Neuropeptides are important messenger molecules that occur in a great variety of forms and are implicated in the regulation of critical physiological processes such as diuresis, digestion, development and reproduction [5,6] in insects. In the past several years, new developments in matrix-assisted laser desorption-time-of-flight mass spectrometry (MALDI-TOF MS) have afforded very sensitive de novo sequencing of peptides via direct analysis of single neurosecretory organs or nerves, including those of insects [12,15,16] via the post-source decay (PSD) technique. Alternatively, sensitive electrospray ionization (ESI) coupled with tandem MS has resulted in the identification of a number of novel neuropeptides [1,17,19,21,22]. The amount of material needed for ESI MS experiments is usually larger than the amount necessary for MALDI MS, since the peptides have to be extracted from the tissue prior to analysis. Both techniques, however, alleviate the need for large numbers of specimens and the time-consuming and expensive efforts required to isolate and determine the primary structure of neuropeptides via traditional chromatographic and chemical sequencing techniques [3,7,25]. These MS techniques have also made it possible to make detailed comparisons of the peptide patterns or profiles (i.e., the peptidomes) of closely related insect species [2,21]. Nonetheless, prior to this study, MS analysis of insect neuropeptides has failed to distinguish between the isomers Leu

* Corresponding author. Tel.: +1 979 260 9315; fax: +1 979 260 9377.
E-mail address: nachman@tamu.edu (R.J. Nachman).

0196-9781/$ – see front matter © 2005 Published by Elsevier Inc.
doi:10.1016/j.peptides.2005.04.024

Report Documentation Page

Form Approved
OMB No. 0704-0188

1. REPORT DATE	2. REPORT TYPE	3. DATES COVERED
27 APR 2005		**00-00-2005 to 00-00-2005**

4. TITLE AND SUBTITLE	5a. CONTRACT NUMBER
Mass spectrometric assignment of Leu/Ile in neuropeptides from single neurohemal organ preparations of insects	5b. GRANT NUMBER
	5c. PROGRAM ELEMENT NUMBER
6. AUTHOR(S)	5d. PROJECT NUMBER
	5e. TASK NUMBER
	5f. WORK UNIT NUMBER
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) **U.S. Department of Agriculture,Areawide Pest Management Research,2881 F/B Road,College Station,TX,77845**	8. PERFORMING ORGANIZATION REPORT NUMBER
9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES)	10. SPONSOR/MONITOR'S ACRONYM(S)
	11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION/AVAILABILITY STATEMENT
Approved for public release; distribution unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT
see report

15. SUBJECT TERMS

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			
unclassified	**unclassified**	**unclassified**	**Same as Report (SAR)**	**6**	

and Ile, which feature identical masses. These studies were limited to low energy fragmentations of the ion of interest. A primary limitation of PSD peptide sequencing is that the internal energies of the $[M+H]^+$ ions are not sufficient to yield the side-chain cleavages necessary to distinguish Leu and Ile.

However, recent innovations in MALDI-TOF MS have allowed analysis of collision-induced fragmentation of primary ions of peptides that reveal unique patterns for the side-chains of Leu and Ile [10,11].

In this study, we utilize MALDI-TOF/TOF tandem MS to provide unambiguous assignment of internal Leu/Ile positions of PVK/CAP2b (periviscerokinin/cardioacceleratory peptide 2b) neuropeptides from single neurohemal organ preparations of adults of the housefly *Musca domestica* and flesh fly *Neobellieria bulleria*. PVKs are typical of the abdominal neurohemal system of insects [26], usually stored in abdominal perisympathetic organs. The PVK/CAP2b class of neuropeptides has been shown to elicit both myotropic activity and stimulation of Malpighian tubule fluid secretion in insects (see Ref. [26]), physiological processes critical to survival.

2. Materials and methods

2.1. Insects

Houseflies (*M. domestica*) were obtained as pupae from the Knipling-Bushland U.S. Livestock Insects Laboratory in Kerrville, TX, and kept in cages at 26 °C. Flesh flies (*Neobellieria bullata*) were obtained in colonies maintained at the Areawide Pest Management Research Unit, Southern Plains Agricultural Research Center in College Station, TX. This flesh fly colony was propagated by placing pupae in a cage containing water, sugar cubes and a powdered milk:sugar mixture (50:50), and cultured at 26 °C. After emergence of the adults, the flies were fed beef liver provided fresh each day for a total of 4 days.

2.2. Matrix-assisted laser desorption ionization time-of-flight tandem mass spectrometry (MALDI-TOF/TOF MS)

Dissection and sample preparation were performed as described in Predel and Gäde [16]. MALDI analysis was performed on the ABI4700 proteomics analyzer (Applied Biosystems, Framingham, MA) [11]. Due to the nature of the samples all acquisitions were taken in manual mode. Initially, the instrument was operated in reflectron mode, in order to determine the parent masses. The laser intensity was set just above the threshold required to ionize the neuropeptides. For the tandem MS experiments, the acceleration was 1 kV in all cases, and the laser intensity was increased by 10%. The number of laser shots used to obtain a spectrum varied from 500 to 5000, depending on signal quality. In order to change the net amount of collisions to the primary ions in the collision-induced dissociation (CID) experiment, the collision cell gas (atmospheric air) pressure was increased. All three gas pressures settings (none, medium and high) available were employed. The instrument is operated in PSD mode when no collision gas is used. The true pressure within the collision cell cannot be measured. The fragmentation patterns from these three different settings were used to determine the sequence of the peptide. The fragmentation data obtained in these experiments was handled using the Applied Biosystems Data Explorer® software package.

2.3. Peptide synthesis

Musdo-PVK-1 (AGGTSG**L**YAFPRVa), the I analog of Musdo-PVK-1, and Musdo-PVK-2 (AS**L**FNAPRVa) were synthesized via Fmoc methodology on Rink Amide resin (Novabiochem, San Diego, CA) using Fmoc protected amino acids (Advanced Chemtech, Louisville, KY) on an ABI 433A peptide synthesizer (Applied Biosystems, Foster City, CA) under previously described conditions [13]. Crude products were purified on a Waters C_{18} Sep Pak cartridge and a Delta Pak C_{18} reverse-phase column (8 mm × 100 mm, 15 μm particle size and 100 A pore size) on a Waters 510 HPLC controlled with a Millennium 2010 chromatography manager system (Waters, Milford, MA) with detection at 214 nm at ambient temperature. Solvent A = 0.1% aqueous trifluoroacetic acid (TFA) and Solvent B = 80% aqueous acetonitrile containing 0.1% TFA. *Conditions*: Initial solvent consisting of 20% B was followed by the Waters linear program to 100% B over 40 min; flow rate, 2 ml/min. Delta Pak C_{18} retention times: Musdo-PVK-1 (AGGTSG**L**YAFPRVa), 11.5 min; [**I**^{7}]Musdo-PVK-1 (AGGTSG**I**YAFPRVa), 10.0 min; Musdo-PVK-2 (AS**L**FNAPRVa), 12.5 min. The peptides were further purified on a Waters Protein Pak I125 column (7.8 mm × 300 mm) (Milligen Corp., Milford, MA). *Conditions*: Flow rate: 2.0 ml/min; Solvent A = 95% acetonitrile made to 0.01% TFA; Solvent B = 50% aqueous acetonitrile made to 0.01% TFA; 100% A isocratic for 4 min, then a linear program to 100% B over 80 min. WatPro retention time: Musdo-PVK-1 (AGGTSG**L**YAFPRVa), 9.0 min; [I^7]Musdo-PVK-1 (AGGTSG**I**YAFPRVa), 9.5 min; Musdo-PVK-2 (AS**L**FNAPRVa), 8.5 min. Amino acid analysis was carried out under previously reported conditions [13] and used to quantify the peptide and to confirm identity, leading to the following analyses: Musdo-PVK-1 (AGGTSG**L**YAFPRVa): A[1.9], F[1.0], G[2.6], L[1.0], P[1.1], R[1.0], S[0.9], T[1.0], V[1.1], Y[1.0]; [I^7]Musdo-PVK-1 (AGGTSG**I**YAFPRVa), A[1.9], F[1.0], G[2.6], I[0.9], P[1.1], R[1.0], S[0.9], T[1.0], V[1.0], Y[0.9]; Musdo-PVK-2 (AS**L**FNAPRVa): A[2.0], F[1.0], L[0.9], N[0.9], P[1.1], R[0.9], S[0.9], V[1.7]. The identity of the peptide analog was confirmed via MALDI-TOF MS on a Kratos Kompact Probe MALDI-TOF MS machine (Kratos Analytical Ltd., Manchester, UK) with the presence of the following molecular ions ($M+H^+$):

Musdo-PVK-1 (AGGTSG**L**YAFPRVa), 1294.4 [M + H$^+$]; [I^7]Musdo-PVK-1 (AGGTSG**I**YAFPRVa), 1294.7 [M + H$^+$]; Musdo-PVK-2 (AS**L**FNAPRVa), 973.9 [M + H$^+$].

2.4. Isolated Malpighian tubule preparations

Fluid secretion from isolated Malpighian tubules was measured using the "Ramsay assay" as previously described for housefly tubules [7]. Tubules were dissected under *Musca* saline [7] and the posterior pair was transferred to small (10 μl) drops of bathing fluid (a 1:1 mixture of saline and Schneider's *Drosophila* medium) beneath water-saturated liquid paraffin in a Sylgard^{TM} lined Petri dish. The tubules were allowed to equilibrate for 1 h before being challenged with test peptides. Urine escaped from the cut end of the tubule, which was pulled out into the liquid paraffin. Drops of urine were collected at 15–20 min intervals and their diameter (d) measured as they rested on the Sylgard base of the Petri dish using a Wild digital (MMS235) eyepiece micrometer. Urine volume was calculated as $\pi d^3/6$ and the rate of secretion obtained by dividing the secreted volume by the collection period. Results are presented as the mean ± S.E.M. for the number of determinations (N) shown in parentheses. Dose–response curves were prepared using the computer program GraphPad Prism Version 3.02 (GraphPad Software, San Diego, CA) and individual curves were compared as described on the company website (http://www.graphpad.com). Where appropriate, statistical tests were carried out using GraphPad Instat Version 3.01 with $P < 0.05$ being accepted as the standard of significance.

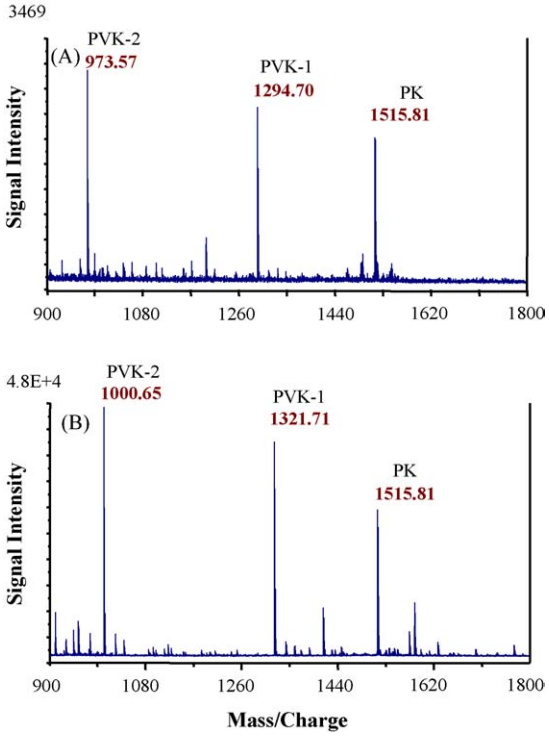

Fig. 1. MALDI-TOF mass spectra of preparations of the posterior abdominal dorsal sheath of the ventral nerve cord of the housefly *M. domestica* (A) and the grey flesh fly *N. bullata* (B). The dorsal sheath represents a neurohemal release site homologous to perisympathetic organs which become incorporated into the dorsal ganglionic sheath during the metamorphosis of cycloraphous flies. Abundant mass signals represent products of the respective capa genes.

3. Results

3.1. Determination of PVK/CAP2b sequences in abdominal dorsal sheaths of flies via MALDI-TOF/TOF mass spectrometry

Direct analysis of abdominal dorsal sheath tissues from the housefly *M. domestica* and flesh fly *N. bullata* were conducted via MALDI-TOF/TOF MS. Illustrated in Fig. 1 are the initial MALDI-TOF spectra of the preparations of these two flies taken under normal conditions that feature the parent ions of the capa gene products (PVK/CAP2b and pyrokinin). Following this, the spectra were retaken under conditions of high gas pressure to promote collision-induced fragmentation of the primary ions of the PVK/CAP2b peptides that reveal unique patterns for the side-chains of Leu and Ile [10,11]. As illustrated in Fig. 2, fragments of native Musdo-PVK-1 of the housefly include a prominent 'w7a' fragment ion at m/z 805.49 (Fig. 2A), indicative of Leu. Indeed, the spectrum of the synthetic version of Musdo-PVK-1 containing Leu at position 7 taken under the same conditions is virtually identical (w7a = m/z 805.49) (Fig. 2B). In contrast, a spectrum of the synthetic version of Musdo-PVK-1 containing Ile at position 7 under conditions of high gas pressure shows a 'w7a' fragment ion at 819.43 along with a 'v-ion' at 806.41 and a 'wb-ion' at 833.46 (Fig. 2C). In Fig. 2D, a spectrum of the Ile analog demonstrates that when the collision gas is turned off, a spectrum results in which the w7a, v and wb-ions disappear and Ile cannot be distinguished from Leu. Thus, Musdo-PVK-1 can be unambiguously assigned the sequence AGGTSG**L**YAFPRVa (Table 1). Using the same single organ preparations, the sequences of Musdo-PVK-2 and Neobu-PVK-1 could also be unambiguously determined under conditions of high gas pressure to be AS**L**FNAPRVa and NGGTSG**L**FAFPRVa (Table 1), respectively. The sequence of Neobu-PVK-2 can be narrowed to AG**LI**VYPR[**L/I**]a (Table 1), in which the internal aliphatic residues can be determined unambiguously.

Table 1
Amino acid sequences of PVK/CAP2b peptides native to the housefly (*M. domestica*) and flesh fly (*N. bullata*) determined by MALDI-TOF–TOF tandem mass spectrometry

Species	PVK-1	PVK-2
M. domestica	AGGTSG**L**YAFPRVa	AS**L**FNAPRVa
N. bullata	NGGTSG**L**FAFPRVa	AG**LI**VYPR[**L/I**]a[a]

[a] MALDI-TOF–TOF tandem mass spectrometry cannot distinguish between Leu and Ile at a C-terminal position.

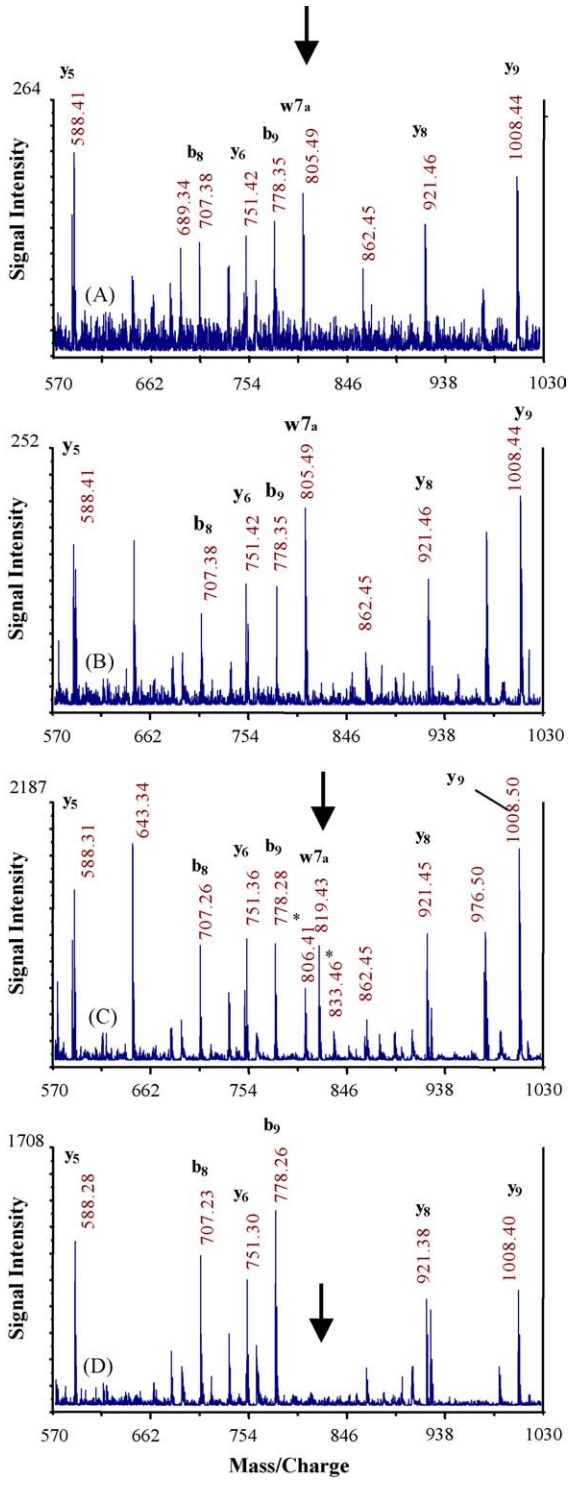

Fig. 2. MALDI-TOF/TOF tandem mass spectra of (A) native Musdo-PVK-1 under conditions of high gas pressure (arrow identifies the 'w7a' fragment ion at 805.49), (B) the synthetic variant with Leu under conditions of high gas pressure CID ('w7a' fragment at 805.49), (C) the synthetic variant with Ile under conditions of high gas pressure CID (arrow identifies 'w7a' fragment and the asterisk denotes the accompanying 'v-ion' at 806.41 and 'wb-ion' at 833.46) and (D) the synthetic Ile variant when the gas source is turned off ('w7a', 'v' and 'wb'-ions disappear). The mass spectra of the native and synthetic Leu variant are essentially identical and differ from that of the synthetic Ile variant.

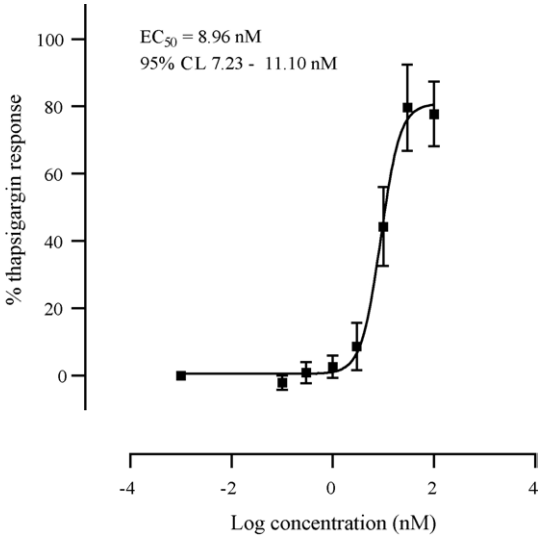

Fig. 3. Dose–response curve for the effect of native Musdo-PVK-1 on fluid secretion by Malpighian tubules from the housefly *M. domestica*. Data points show the means ± S.E. of 6–14 determinations.

Table 2
Fluid secretion activity of native Musca-PVK-1 and Musca-PVK-2 on housefly (*M. domestica*) Malpighian tubules

Peptide	Sequence	Fluid secretion[a] [EC_{50}] (nM)
Musca-PVK-1	AGGTSG**L**YAFPRVa	9 (95% CL: 7.2–11.1)
Musca-PVK-1[I^7]	AGGTSG**I**YAFPRVa	2 (95% CL: 0.3–7.1)
Musca-PVK-2	AS**L**FNAPRVa	102 (95% CL: 96–109)
Manse-CAP2b	pE**L**YAFPRVa	53 (95% CL: 39–72)[b]

[a] Values in brackets, ( ), represent the 95% confidence limit (CL).
[b] Coast and Garside, unpublished data.

3.2. Fluid secretion activity of PVK/CAP2b sequences on housefly Malpighian tubules

Synthetic replicates of the two PVK sequences native to the housefly were evaluated for diuretic activity on isolated Malpighian tubules. As can be seen in Fig. 3 and Table 2, the two analogs Musdo-PVK-1 and Musdo-PVK-2 stimulated housefly Malpighian tubule fluid secretion with observed EC_{50} values of 9 and 102 nM, respectively. As a comparison, the diuretic activity of the unnatural Ile analog of Musca-PVK-1 ([I^7]Musca-PVK-1) proved to be more potent than the native peptide, with an EC_{50} of 2 nM.

4. Discussion

PVK/CAP2b peptides have been found in the housefly *M. domestica* and flesh fly *N. bullata* via MALDI-TOF mass spectrometric analysis of preparations of neurohemal tissues [21]. PVK/CAP2b peptides of insects are typical of neurosecretory neurons in the abdominal ganglia and are accumulated in perisympathetic organs until release. Larval perisympathetic organs of cycloraphous Diptera, however, become incorporated into the dorsal ganglionic sheath [14]

during the metamorphosis. The abdominal dorsal sheath, which was dissected in this study, therefore contains relatively large amounts of peptidergic neurohormones. The molecular ions and incomplete sequences observed in earlier mass spectrometric studies on the housefly and flesh fly are Musdo-PVK-1 (AGGTSG[**L/I**]YAFPRVa; *m*/*z* 1294.7) and Musdo-PVK-2 (AS[**L/I**]FNAPRVa; *m*/*z* 973.6); Neobu-PVK-1 (NGGTSG[**L/I**]FAPRVa; *m*/*z* 1321.7) and Neobu-PVK-2 (AG[**L/IL/I**]VYPR[**L/I**]a; *m*/*z* 1000.7). Using the fragment ion patterns from previous MS/MS experiments, one could not distinguish between the conservative Leu and Ile isomers. From the initial mass spectrometric investigation, however, the specific identity of the native peptides has been narrowed to two possible sequences for Musdo-PVK-1, Musdo-PVK-2 and Neobu PVK-1; eight possible sequences for Neobu-PVK-2 [21,25]. Similar results were obtained when single neurohemal organs (abdominal dorsal ganglionic sheaths) were studied on a MALDI-TOF–TOF mass spectrometer (Fig. 1). However, when the peptides were fragmented under conditions of high collision energy, the collision-induced fragments reveal distinct side-chain fragmentations. Musdo-PVK-1 demonstrated a fragmentation pattern indicative of a Leu in position 7, and this was confirmed via fragmentation studies done on a synthetic replicate containing Leu. The synthetic replicate containing Ile at this position demonstrated a different fragmentation pattern (see Section 3). Thus, Musdo-PVK-1 can be unambiguously assigned the sequence AGGTSG**L**YAFPRVa (Table 1). Using the single organ preparations, the sequences of Musdo-PVK-2 and Neobu-PVK-1 could also be unambiguously determined under conditions of high collision energy to be AS**L**FNAPRVa and NGGTSG**L**FAFPRVa (Table 1), respectively. The sequence of Neobu-PVK-2 can be narrowed to AG**LI**VYPR[**L/I**]a (Table 1), in which the internal aliphatic residues can be determined unambiguously. This is the first report using MALDI-TOF/TOF tandem mass spectrometry to distinguish between Leu and Ile of peptides in tissue of an insect and/or arthropod specimen.

It is clear that the Leu at the position located 7 residues from the C-terminus in these four dipteran PVK/CAP2b sequences is conserved within and across species (Table 1). Leu at this specific position is typical of other PVK/CAP2b of insects that were sequenced by Edman degradation in earlier studies [4,8,17,18,20] or for which genes have been published already [9,23,24]. While the internal L/I pairs could be unambiguously assigned, tandem mass spectrometry cannot distinguish between Leu and Ile at the C-terminus, as is the case with Neobu-PVK-2. Nonetheless, the possible sequences for Neobu-PVK-2 have been narrowed from eight to two using the analysis of spectra from MALDI-TOF–TOF tandem mass spectrometry.

The diuretic activity of the peptide sequences native to the housefly *M. domestica* on housefly Malpighian tubules was compared. The diuretic activity of Musdo-PVK-1 is greater than Musdo-PVK-2 by an order of magnitude. The diuretic activity of Musdo-PVK-1 is also significantly greater (five-fold) than Manse-CAP2b (pELYAFPRVa; EC_{50} = 53 nM [Coast, personal communication]) (Table 2), the *Manduca sexta* (Lepidopera) peptide first used to demonstrate that members of this family can stimulate Malpighian tubule fluid secretion in the housefly. Manse-CAP2b and Musdo-PVK-1 share an identical C-terminal heptapeptide sequence. Musdo-PVK-2 is less active than Manse-CAP2b by a factor of two, likely because of considerable sequence differences in the C-terminal heptapeptide core portions of the two peptides (i.e., YAF versus FNA). Interestingly, the diuretic activity of the unnatural Ile analog of Musca-PVK-1 ([I^7]Musca-PVK-1) (Table 2) proved to be 4.5 times more potent than the native Leu-containing peptide, with an EC_{50} of 2 nM. This should be taken as a cautionary note, as it demonstrates that the most active analog does not represent the native sequence and serves as a reminder that the potency values in a biological assay cannot be relied upon to predict the native sequence.

In summary, MALDI-TOF/TOF tandem mass spectrometry has been used for the first time to unambiguously distinguish between internal Leu and Ile in insect neuropeptides via direct analysis of nerve tissue. The work underscores the power of this technique in cataloguing the peptidomes of related species for systematic/taxonomic purposes and in the identification of the specific sequences of insect neuropeptides that are implicated in the regulation of critical physiological processes.

Acknowledgements

This study was supported by a Binational Agricultural Research and Development Grant (BARD #IS-3356-02) (RJN), a grant from Deutsche Forschungsgemeinschaft (Predel 595/6-1), a Collaborative Research Grant (#LST.CLG.979226) from the North Atlantic Treaty Organization (NATO) (RJN, GMC), a grant from the USDA/DOD DWFP Initiative (#0500-32000-001-01R) (RJN), and grants provided to the Laboratory for Biological Mass Spectrometry at Texas A&M College Station: R01 RR019587 NIH and DE-FG02-04ER15520 DOE. In addition, we acknowledge the capable technical assistance of Allison Strey, David Rigolout and Pawel Zubrzak of the Areawide Pest Management Research Unit, Southern Plains Agricultural Research Center.

References

[1] Baggerman G, Cerstiaen A, De Loof A, Schoofs L. Peptidomics of the larval *Drosophila melanogaster* central nervous system. J Biol Chem 2002;277:40368–74.

[2] Clynen E, Baggerman G, Huybrechts J, Vanden Bosch L, De Loof A, Schoofs L. Peptidomics of the locust corpora allata: identification of novel pyrokinins (FXPRLamides). Peptides 2003;24:1493–500.

[3] Clynen E, Baggerman G, Veelaert A, Cerstiaens A, Van der Horst D, Harthoorn L, et al. Peptidomics of the pars intercerebralis-corpus cardiacum complex of the migratory locust, *Locusta migratoria*. Eur J Biochem 2001;268:1929–39.

[4] Clynen E, Huybrechts J, De Loof A, Schoofs L. Mass spectrometric analysis of the perisympathetic organs in locusts: identification of novel periviscerokinins. Biochem Biophys Res Commun 2003;300:422–8.
[5] Coast GM. The neuroendocrine regulation of salt and water balance in insects. Zoology (ZACS) 2001;103:179–88.
[6] Gäde G. The explosion of structural information on insect neuropeptides. In: Herz W, Kirby GW, Moore RE, Steglich W, Tamm C, editors. Progress in the chemistry of organic natural products. Vienna: Springer-Verlag; 1997. p. 1–128.
[7] Holman GM, Nachman RJ, Coast GM. Isolation, characterization and biological activity of a diuretic myokinin neuropeptide from the housefly, *Musca domestica*. Peptides 1999;20:1–10.
[8] Huesmann GR, Cheung CC, Loi PK, Lee TD, Swiederek KM, Tublitz NJ. Amino acid sequence of CAP_{2b}, an insect cardioacceleratory peptide from the tobacco hawkmoth *Manduca sexta*. FEBS Lett 1995;371:311–4.
[9] Loi PK, Tublitz NJ. Sequence and expression of the CAPA/CAP2b gene in the tobacco hawkmoth, *Manduca sexta*. J Exp Biol 2004;207:3681–91.
[10] Macht M, Asperger A, Deinenger SO. Comparison of laser-induced dissociation and high-energy collision-induced dissociation using matrix-assisted laser desorption/ionization tandem time-of-flight (MALDI-TOF/TOF) for protein and peptide identification. Rapid Commun Mass Spectrom 2004;18:2093–105.
[11] Medzihradszky KF, Campbell JM, Baldwin MA, Falick AM, Juhasz P, Vestal ML, et al. The characteristics of peptide collision-induced dissociation using a high-performance MALDI-TOF/TOF tandem mass spectrometer. Anal Chem 2000;72:552–8.
[12] Nachman RJ, Coast GM, Tichy SE, Russell DH, Miller JA, Predel R. Occurrence of insect kinins in the flesh fly, stable fly and horn fly-mass spectrometric identification from single nerves and diuretic activity. Peptides 2002;23:1885–94.
[13] Nachman RJ, Isaac RE, Coast GM, Holman GM. Aib-containing analogues of the insect kinin neuropeptide family demonstrate resistance to an insect angiotensin-converting enzyme and potent diuretic activity. Peptides 1997;18:53–7.
[14] Nässel DR, Ohlsson LG, Cantera R. Metamorphosis of identified neurons innervating thoracic neurohemal organs in the blowfly-transformation of cholecystokinin like immunoreactive neurons. J Comp Neurol 1988;267:343–56.
[15] Predel R, Gäde G. Identification of the abundant neuropeptide from abdominal perisympathetic organs of locusts. Peptides 2002;23:621–8.
[16] Predel R, Gäde G. Peptidomics of neurohemal organs from species of the cockroach family Blattidae: how do neuropeptides of closely related species differ? Peptides 2005;26:3–9.
[17] Predel R, Kellner R, Baggerman G, Steinmetzer T, Schoofs L. Identification of novel periviscerokinins from single neurohaemal release sites in insects-MS/MS fragmentation complemented by Edman degradation. Eur J Biochem 2000;267:3869–74.
[18] Predel R, Linde D, Rapus J, Vettermann S, Penzlin H. Periviscerokinin (Pea-PVK): a novel myotropic neuropeptide from the perisympathetic organs of the American cockroach. Peptides 1995;16:61–6.
[19] Predel R, Neupert S, Wicher D, Gundel M, Roth S, Derst C. Unique accumulation of neuropeptides in an insect: FMRFamide related peptides in the cockroach, *Periplaneta americana*. Eur J Neurosci 2004;20:1499–513.
[20] Predel R, Rapus J, Eckert M, Holman GM, Nachman RJ, Wang Y, et al. Isolation of periviscerokinin-2 from the abdominal perisympathetic organs of the American cockroach, *Periplaneta americana*. Peptides 1998;19:801–9.
[21] Predel R, Russell WK, Tichy SE, Russell DH, Nachman RJ. Peptidomics of CNS-associated neurohemal systems of adult *Drosophila melanogaster*: a mass spectrometric survey of peptides from individual flies. Peptides 2003;24:1487–91.
[22] Predel R, Wegener C, Russell WK, Tichy SE, Russell DH, Nachman RJ. Peptidomics of CNS-associated neurohemal systems of adult *Drosophila melanogaster*: a mass spectrometric survey of peptides from individual flies. J Comp Neurol 2004;474:379–92.
[23] Riehle MA, Garczynski SF, Crim JW, Hill CA, Brown MR. Neuropeptides and peptide hormones in *Anopheles gambiae*. Science 2002;298:172–5.
[24] Vanden Broeck J. Neuropeptides and their precursors in the fruitfly, *Drosophila melanogaster*. Peptides 2001;22:241–54.
[25] Verleyen P, Huybrechts J, Sas F, Clynen E, Baggerman G, DeLoof A, et al. Neuropeptidomics of the grey flesh fly, *Neobellieria bullata*. Biochem Biophys Res Commun 2004;316:763–70.
[26] Wegener C, Herbert Z, Eckert M, Predel R. The periviscerokinin (PVK) peptide family in insects: evidence for the inclusion of CAP(2b) as a PVK family member. Peptides 2002;23:605–11.